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Full Length Research Article

Partial Purification and Characterization of Extracellular Protease from *Pedicoccus acidilactici*

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ABSTRACT: Microbial proteases have wide industrial applications and proteases of the lactic acid bacteria (LAB) have received special attention because of their importance in the food and dairy industry. Of all the LAB, the proteolytic system of the pediococci is the least studied. Therefore, this study was aimed at characterizing and purifying the protease produced by *Pediococcus acidilactici* grown in de Man, Rogosa and Sharpe (MRS) broth. Casein concentration of 2% (w/v) and 2.5 ml of the crude enzyme were optimal for the activity of the protease. The crude protease had temperature and pH optima of 28 ^{o}C and 4.0 respectively thus indicating that the enzyme is a mesophilic and acidic protease. Purification of the enzyme by gel filtration chromatography on Sephadex G75 following ammonium sulphate precipitation gave 2.26 fold increase in purification with specific activity of 46.13 units/mg protein while purification on Sephadex CM50 resulted in reduced purification fold (1.24 - 1.59) with specific activity ranging between 25.39 - 32.54 units/mg protein. The protease showed a characteristic band on SDS-PAGE and the molecular weight was estimated between 45 and 66 kDa. The potential applications of the protease are discussed.
Keywords: *Protease, lactic acid bacteria, Pediococcus acidilactici,* enzyme purification.

INTRODUCTION

Proteases are hydrolytic enzymes which release peptides and amino acids from protein molecules. They have wide applications in various industries such as pharmaceutical, leather, food and detergent industries (Rao *et al*., 1998). They also find usage in research. They constitute one of the largest groups of industrial enzymes and account for up to 60% of the total global sale of enzymes (Joshi and Satyanarayana, 2013). Proteases can be sourced from animals, plants, viruses, bacteria and fungi. Microbial proteases are more desired for industrial applications due to the flexibility of microbes to genetic manipulation and the fact that microbial proteases possess almost all the characteristics needed for biotechnological application (Rao *et al*., 1998).

The proteolytic system of lactic acid bacteria (LAB) has received special interest because of the role of LAB in the food and dairy industry. Since LAB are unable to synthesize amino acids required for their metabolism, they rely on an active system of proteolytic enzymes which enables them to utilize the nitrogen sources present in their environments (Pritchard and Coolbear, 1993). Besides enabling them to grow in environments such as milk, the proteolytic system of LAB also confers organoleptic improvements in fermented foods for which they act as starters (Savijoki *et al*., 2006).
Proteases of other LAB members such as the lactococci, lactobacilli and the streptococci have been well-studied while those of the pediococci are less reported in the literature. Strains of *Pediococcus acidilactici* isolated from sausages have been reported to possess protease and peptide-hydrolyzing enzymes (Benito *et al*., 2007). The production of protease by *P. acidilactici* is influenced by several environmental factors. Oke and Odebisi (2012) reported that the optimal conditions for protease production by the organism were centrifugation pretreatment of 3500 rpm, pH 6.5 and water activity of 0.99.

The suitability of an enzyme for industrial processes depends on its unique characteristics such as optimum temperature, pH, mode of action, etc. (Zhang *et al*., 2011). Therefore, characterization and purification of an enzyme is highly important in appreciating its potential uses. A better understanding of the protease produced by *P. acidilactici* would allow for further improvements in its use as starter culture in meats and other fermentations and also enable us to identify other potential industrial applications of the enzyme.

MATERIALS AND METHODS

Source of organism

P. acidilactici was obtained from the culture collection of the Department of Microbiology, University of Ibadan, Nigeria. The organism was previously isolated from refrigerated meat samples (Duyilemi, O.P.; personal communication, September 28, 2013). Pure cultures of the organism were maintained on MRS

(Oxoid, UK) agar slants at 4 °C. All reagents used were of analytical grade.

Protease production

Enzyme production was carried out by inoculating 250 ml of sterile MRS broth (Oxoid, UK) (pH 6.5) with *P. acidilactici*. The culture was incubated for 24 hours at 35 °C after which it was centrifuged at 10000 rpm for 20 minutes. The cell-free supernatant was obtained as the crude enzyme and was used for subsequent studies.

Characterization of protease

The crude (cell-free) enzyme extract obtained earlier was characterized by studying the effect of substrate concentration, amount of crude enzyme, temperature and pH on enzyme activity using casein (Difco Laboratories, USA) as substrate.

Protease activity assay and protein estimation

All protease activity assays were done using the modified method of Xiong *et al*. (2007). Except where otherwise stated, 1% casein solution prepared in 0.1M citrate phosphate buffer (pH 5.5) was used as substrate. The solution was heat-denatured at 100 °C for 15 minutes and then allowed to cool (Kunitz, 1947). The enzyme extract (0.5 ml) was thoroughly mixed with the substrate solution (1 ml) and the reaction mixture was incubated at 28 °C for 1 hour. The reaction was terminated by the addition of 3 ml of cold 10% tricholoroacetic acid (TCA) and the tubes were allowed to stand for one hour at 2 °C to allow for precipitation of undigested protein. For the control experiments, 0.5 ml of the enzyme extract was first incubated at 28 °C for 1 hour before the addition of TCA and 1% casein. The reaction mixtures were centrifuged at 10000 rpm for 5 minutes at 4 °C. Optical density readings of the decanted supernatant were measured at 660 nm. One unit of protease activity was defined as the amount of enzyme that released 1 µg tyrosine per ml per minute from casein under the specified assay conditions Protein estimation was done using the method of Lowry *et al*. (1951).

Effect of substrate concentration on protease activity:

Varying concentrations (1 – 5% w/v) of casein were prepared in 0.1M citrate phosphate buffer (pH 5.5). The casein solutions were heat-denatured at 100 °C for 15 minutes in a water bath and were allowed to cool. Protease assay was then carried out using the different substrate concentrations.

Effect of amount of crude enzyme on protease activity:

One percent (1% w/v) casein solution was prepared in 0.1M citrate phosphate buffer (pH 5.5). The casein solution was heat-denatured at 100 °C for 15 minutes in a water bath and allowed to cool. The amount of the crude enzyme extract was varied at 0.5 – 2.5 ml. Protease assay was then carried out using different volumes (0.5 – 2.5 ml) of the crude enzyme extract.

Effect of temperature on protease activity:

One percent casein solution was prepared as described earlier. Protease assay was carried out by incubating 0.5 ml of the enzyme extract with 1 ml of the substrate for 1 hour at 4, 20, 28, 35 and 40 °C.

Effect of pH on protease activity:

One percent (1% w/v) casein solution was prepared in 0.1M citrate phosphate buffer at pH values of 4.0, 4.5, 5.0, 5.5 and 6.0. The casein solutions were then heat-denatured at 100 °C for 15 minutes and allowed to cool. Protease assay was carried out using the prepared casein solutions as substrate.

Purification of protease

Ammonium sulphate precipitation:

The culture broth was centrifuged at 10000 rpm for 20 minutes and the supernatant was collected. The enzyme in the supernatant was precipitated by the addition of solid ammonium sulphate up to 80% saturation level (Dixon and Webb, 1971). The mixture was left to stand at 4 °C for 24 hours and was centrifuged at 10000 rpm for 15 minutes. The final precipitate was dissolved in 90 ml of 0.1M citrate phosphate buffer (pH 5.5) and dialysed with the buffer for 18 hours at 4 °C (Olutiola and Akintunde, 1979).

Gel filtration and ion exchange chromatography

The dialysate was applied to a Sephadex G75 (Sigma-Aldrich, USA) column (640 mm x 25 mm) and eluted with 0.1M citrate phosphate buffer (pH 5.5). The eluted fractions were collected in 5 ml calibrated tubes each of which was analysed for protease activity. The active fractions were pooled and applied to a Sephadex CM50 (Sigma-Aldrich, USA) column (640 mm x 25 mm) and eluted with the same buffer. Eluted fractions were collected in calibrated 5 ml tubes and each of the tubes was analysed for protease activity. The active fractions were collected, pooled together and used for molecular weight determination.

Determination of molecular weight of purified protease

The final pooled sample was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a discontinuous gradient gel buffer system as described by Laemmli (1970) and (Filho and Moreira, 1978). Estimation of the unknown molecular weight was done by comparison with the gel migration distances of the following standard molecular weight markers (Sigma-Aldrich, USA): aprotinin (6.5 kDa), α-lactalbumin (14.2 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), glutamate dehydrogenase (55 kDa), bovine serum albumin (66 kDa), fructose-6-phosphate kinase (84 kDa), phosphorylase (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa)(Tully *et al*., 2006).

RESULTS AND DISCUSSION

Effect of substrate concentration on protease activity

Determination of the optimal concentration of substrate required is crucial in determining the characteristics of an enzyme. Generally in this study, casein was employed as substrate for all the reactions. Maximum activity of *P. acidilactici* protease was obtained at substrate concentration of 2% (Figure 1).

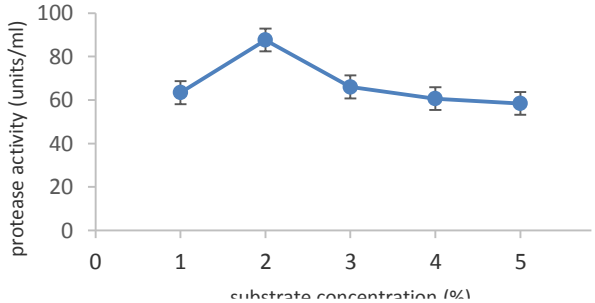

Figure 1: Effect of substrate concentration on protease activity of *P. acidilactici*.

This is similar to the findings of Akinkugbe and Onilude (2013) who reported that protease from *Lactobacillus acidophilus* had maximum activity at 2% casein concentration. Gerze *et al*. (2005) obtained a slightly lesser value (1.2%) for protease produced by *Bacillus subtilis megaterium* (BSM) in their study on the effect of casein concentration and reaction times on protease activity by the bacterium.

Effect of amount of crude enzyme on protease activity

Figure 2 shows the results of tests to determine the effect of the amount of crude enzyme on protease activity of *P. acidilactici* protease. The highest activity was recorded with 2.5 ml of the crude enzyme. Akinkugbe and Onilude (2013) obtained a similar result of optimum crude enzyme amount (2.5 ml) for protease activity of *Streptococcus lactis* and a lower value of 0.5 ml for protease produced by *L. acidophilus*.

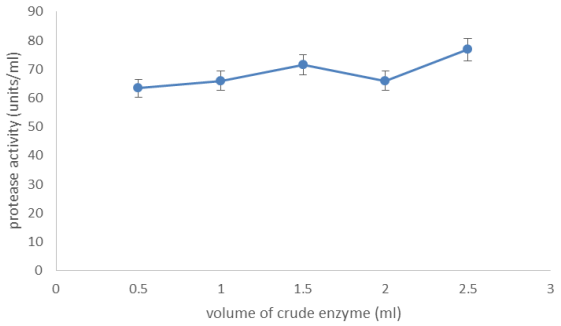

Figure 2: Effect of amount of crude enzyme on protease activity of *P. acidilactici*

Effect of temperature on protease activity

The protease from *P. acidilactici* in this study displayed optimum activity at a temperature of 28 ^{o}C (Figure 3), thus indicating that it is a mesophilic protease. Proteases with similar temperature optimum from other related bacteria have been reported in literature. (Yuan *et al*., 2009) reported that the optimum temperature of action of protease isolated from a LAB, *Enterococcus faecalis* TN-9 was 30 ^{o}C. The enzyme maintained stability as long as the temperature was lower than 45 ^{o}C. In another study, *Streptococcus lactis* and *Lactococcus lactis* both produced proteases which both had maximum activity at 28 ^{o}C and declined activity after 30 ^{o}C (Akinkugbe and Onilude, 2013). The protease in this study was completely inactivated at 37 ^{o}C (Figure 3). A similar result was obtained from the protease of *Xenorhabdus nematophila* which displayed sharp drop in enzyme activity at temperatures above 30 ^{o}C (Mohamed and Hussein, 2007). Enzymes are known to be sensitive to temperature changes and the change in activity of any particular enzyme to such conditions is a distinguishing characteristic of such an enzyme. The use of non-thermophilic, cold-adapted proteases in detergents is desired as it promotes decrease in energy consumption (Joshi and Satyanarayana, 2013).

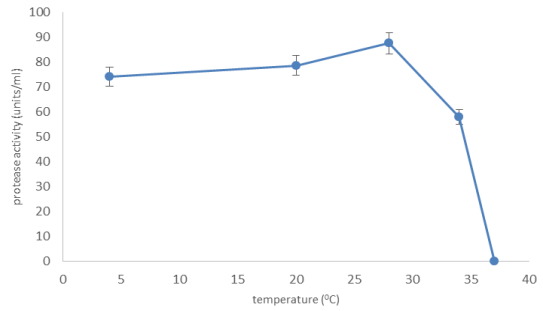

Figure 3: Effect of temperature on activity of *P. acidilactici* protease.

Effect of pH on protease activity

Different enzymes have different pH optimum. From Figure 4, it can be seen that the pH optimum for the protease produced by *P. acidlatici* in this study is 4.0, thus suggesting that it is an acidic protease. Olajuyigbe *et al*. (2004) obtained a protease from *Aspergillus niger* NRRL 1785 which had an optimum pH of 4.0. *P. acidilactici* protease in this study was completely inactivated at pH 6.0.

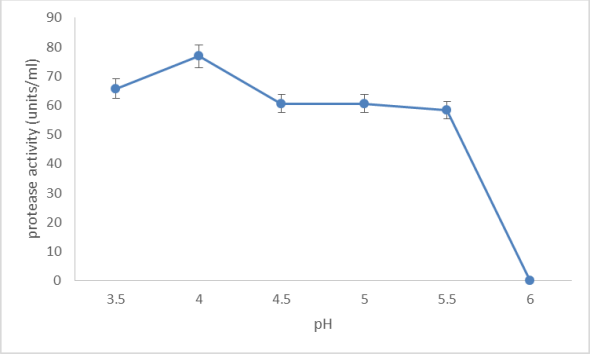

Figure 4: Effect of pH on activity of *P. acidilactici* protease.

This is possibly as a result of the change in the shape and charge properties of the enzyme and substrate. pH change causes alteration of the ionization state of amino acids in a protein which leads to the alteration of the ionic bonds which determine the tertiary structure and charge properties of the protein. This ultimately results in enzyme inactivation or altered substrate recognition (Palmer, 1981).

Acidic proteases are not as commonly found in bacteria as compared to fungi, animal cells and plants (Sumantha *et al*., 2006). Acidic proteases have been isolated from various species of *Aspergillus* (O'Donnell *et al*., 2001; Tremacoldi *et al*., 2004), *Mucor* (Escobar and Barnett, 1993; Fernandez-Lahore *et al*., 1999) and *Rhizopus* (Kumar *et al*., 2005). Fu *et al*. (2008) obtained an acid protease from *Bacillus megaterium* KLP-98 which had an optimum pH of 5.5 and was stable in the pH range of 4.5 – 6.0. Acidic proteases have extensive applications in the dairy industry for the coagulation of milk protein in cheese production (Sumantha *et al*., 2006) and generally in improvement of food flavours (Siala *et al*., 2009).

Purification of protease

It is important to purify proteases to homogeneity in order to study their mechanism of action (Rao *et al*., 1998). Table 1 summarizes the results of purification procedures of protease from the culture supernantant of *P. acidilactici*. The protease was purified by a three-step procedure involving ammonium sulphate treatment, gel filtration chromatography using Sephadex G75 and ion exchange chromatography using Sephadex CM50. Ammonium sulphate precipitation resulted in a 1.48 fold increase in purification with specific activity of 30.31 units/mg protein over the crude enzyme (20.45 units/mg protein). The results show that ammonium sulphate is an effective and simple means of protein separation.

Table 1: Partial purification of protease from *Pediococcus acidilactici.*

Fraction	Total protein (mg/ml)	Total activity (units/ml)	Specific activity (units/ mg protein)	Yield (%)	Purification
Crude extract	279	5705.1	20.45	100	1
Ammonium sulphate precipitation	180	5455.8	30.31	95.63	1.48
Sephadex G75	0.95	43.82	46.13	0.77	2.26
Sephadex CM50					
Pool A	1.05	33.01	31.44	0.58	1.54
Pool B	1.30	33.01	25.39	0.58	1.24
Pool C	1.10	35.79	32.54	0.63	1.59

The increase in yield and specific activity suggests that it is able to fractionate the enzyme from the crude mixture while preserving its activity. This is due to the high solubility of ammonium sulphate which allows for the aggregation and precipitation of proteins in aqueous solutions at different salt concentrations (Wenk and Fernandis, 2007). Purification of the enzyme on Sephadex G75 gave a 2.26 fold increase in purity with a specific activity of 46.13 units/mg protein over the crude extract. Sephadex G75 was able to separate the crude extract into active fractions which resulted in the observed increase in specific activity and purification fold. Very few studies have described the purification of proteases from the pediococci as compared to the number of studies reporting proteolytic activity in the genus. Simitsopoulou *et al*., (1997) purified a 100 kDa intracellular tripeptidase from *P. pentosaceus* K9.2 using a three-step purification protocol involving anion exchange chromatography, gel filtration chromatography and HPLC and achieved up to 755 purification fold increase with specific activity of 1,041 µmol min^{-1} mg^{-1}.

However, the gain in purification fold and specific activity of the enzyme was reversed upon purification on Sephadex CM50. Purification reduced to as low as 1.24 for Pool B while the specific activity reduced to 33.01 units/mg protein for Pools A and B. This is suggests that Sephadex CM50 was not suitable for the protease. This loss in activity could also be as a result of enzyme degradation or due to poor binding and elution as a result of the chosen buffer pH. It is sometimes necessary to add substances such as 1,4-dithiothreitol (DTT) to the chromatography setup so as to protect against loss of activity (e Silvia *et al*., 2011). The low level of purification and specific activity observed after gel filtration and ion exchange chromatography suggests that other purification methods would be more appropriate. Furthermore, the observed results could be due to the poor induction of protease in the organism by casein which was used as the substrate in this study as different substrates induce enzyme production by different levels among bacteria. Further evaluation of other substrates for protease induction in *P. acidilactici* is thus necessary for deriving higher yield of the enzyme.

Molecular weight estimation

Figure 5 shows the electrophoretogram of the purified protease from *P. acidilactici* as determined using SDS-PAGE. The molecular weight of the purified enzyme was estimated between 45 and 66 kDa. Simitsopoulou *et al*., (1997) reported that protease from *P. pentosaceus* K9-2 showed a single band of 45 kDa on SDS-PAGE. Gerze *et al*. (2005) reported a similar result for protease produced by *Bacillus subtilis megaterium*.

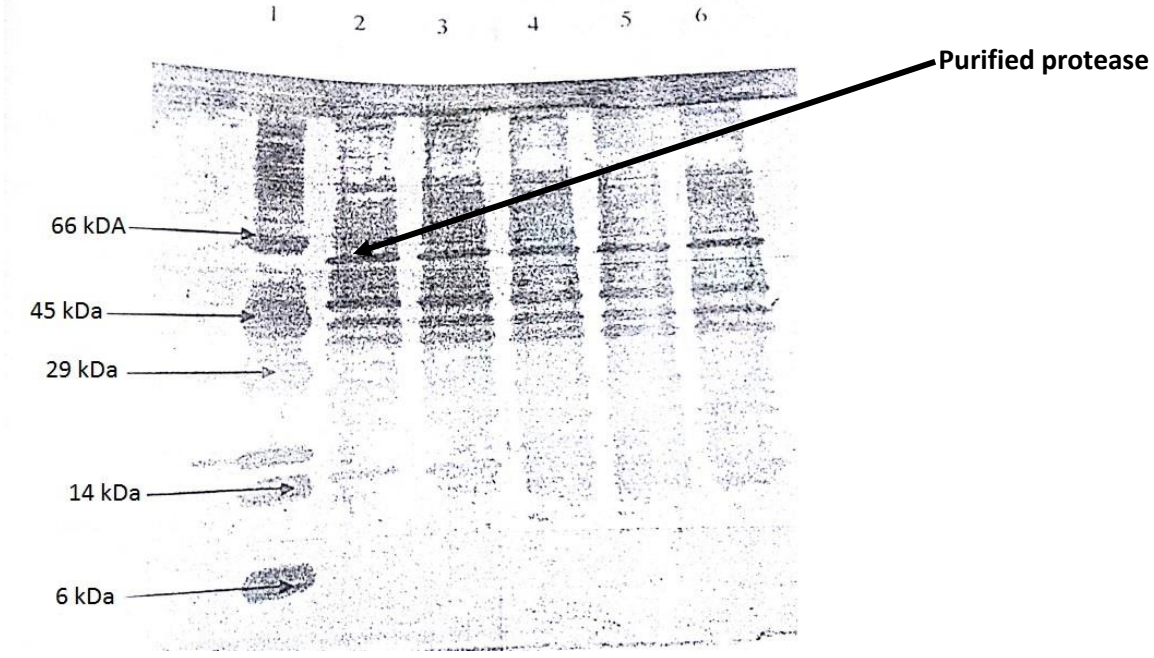

Figure 5: Electrophoretogram of purified protease of *P. acidilactici*.
Lane 1 – molecular weight marker protein standards. Lane 2 to 6 – purified protease.

CONCLUSION

The aim of this study was to isolate, purify and characterize extracellular protease produced by *P. acidilactici*. Activity of the enzyme was found to be maximum at 2% casein concentration and with 2.5 ml volume of the crude of enzyme. Experiments on the temperature dependence of protease activity revealed that the enzyme had optimal activity at 28 ^{o}C which suggests that it is a mesophilic protease. The optimum pH value was 4.0, indicating the acidic nature of the protease. Purification of the enzyme using gel filtration chromatography gave 2.26 fold increase in purity and corresponding increase in specific activity (46.13 units/ mg protein). However, further purification on Sephadex CM50 resulted in a loss in purity and specific activity, indicating that this method is unsuitable for the enzyme. Our results indicate that *P. acidilactici* protease studied in this work is an acidic and mesophilic protease. It has promising potentials for application in processes such as cheese production, improvement of food flavours and non-thermophilic applications such as in detergents for cold washing.

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